Some observations on the possible nutritional significance of vitamin B_{12}- and folate-binding proteins in milk. Absorption of [^{58}Co]cyanocobalamin by suckling piglets

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1. A study was made of the absorption of [^{58}Co]cyanocobalamin in suckling piglets. Cyanocobalamin given at birth and at 7 d of age was efficiently absorbed from the intestine and retained within the body, mostly in the liver. A 10 μg test dose was absorbed no less efficiently than 0·5 μg, despite the virtual absence of intrinsic factor in the gut. In piglets given a 10 μg test dose at different ages between 0·5 and 56 d, there was a marked decrease in the efficiency of retention between about 7 and 21 d of age.

2. Vitamin B_{12}-binding capacity in the gastric mucosa increased with age, from 40 ng at birth to about 2000 ng at 14 d and 7000 ng at 35 d. This binder-protein was largely endogenous, whereas much of the unsaturated binder-protein in intestinal mucosa was apparently derived from milk.

3. The chyme in the stomach and small intestine contained unsaturated binder-protein, partly endogenous and partly deriving from milk, which prevented uptake of added [G-^{3}H]-cyanocobalamin into the 'solids' phase of the intestinal contents. The intestinal chyme contained large numbers ($\log_{10}$ 7·0–9·1/ml) of bacteria, some of which were isolated and shown to take up cyanocobalamin or folic acid or both.

4. The findings are discussed in relation to the concept that for some days or weeks after the cessation of transport of intact protein across the neonatal gut epithelium ('closure'), protein-bound vitamins may continue to be taken up into the epithelial cells and there released for transport into the circulation. It is concluded that unsaturated vitamin-binders may strongly influence the ecology of the intestinal microflora.

The vitamin B_{12} and folate in milk are strongly and specifically bound to minor whey proteins. These binding proteins are generally present in excess; the unsaturated binding capacity varies between milks of different mammalian species. Human milk may bind about 80 ng added cyanocobalalamin/ml, much more than its natural content of vitamin B_{12}, which is about 0·3 ng/ml. Similarly, sow's milk contains about 2 ng vitamin B_{12}/ml but may bind 245 ng added cyanocobalamin/ml. In contrast, cow's milk contains about 3 ng vitamin B_{12}/ml but has little or no capacity to bind added cyanocobalamin (Gregory, Ford & Kon, 1952; Gregory & Holdsworth, 1955*a*, *b*; Ford, 1974). In all three species, the mature milk can bind about 60 ng added folic acid/ml. The presence of these vitamin-binders in milk raises important questions concerning their possible influence on the vitamin economy of the young mammal during the period from birth to weaning, and on the ecology of the intestinal microflora (cf. Ford, 1974).

In the newborn rat the stomach mucosa contains less than 1 % of the intrinsic

factor (IF) activity found in the adult, but the ileum is nevertheless capable of absorbing cyanocobalamin with high efficiency, even when the vitamin is given in excessive amounts (Boass & Hastings Wilson, 1963; Williams & Spray, 1968; Gallagher, 1969). By the time of weaning this capacity of the ileum to absorb cyanocobalamin independently of IF is no longer demonstrable, while IF-mediated absorption has increased. Transmission of passive immunity in the rat is mostly by absorption of the immune globulins from the colostrum and milk and it continues for much of the lactation period. The intact vitamin B_{12}–protein complex probably passes from the gut to the circulation in the same way and represents the predominant mode of vitamin B_{12} uptake. The same may be true for the protein-bound milk folate, which in the newborn goat is efficiently transported intact into the blood plasma, together with the unsaturated folate-binder (Ford, Knaggs, Salter & Scott, 1972). But in the piglet, unlike the rat, absorption of immune globulins decreases rapidly after birth and effectively ceases by the end of the first day. Similarly in the human baby, little or no antibody is absorbed from the colostrum and milk (Brambell, 1970). Thus, in these two species, it seems probable that the protein-bound vitamins are absorbed less directly.

The present paper describes experiments on the absorption and efficiency of retention, and on the partition within the body, of [^{58}Co]cyanocobalamin given to piglets at birth or at ages up to 56 d. The findings are discussed in relation to the amount and the nature of the vitamin B_{12}-binding activity determined in contents and mucosas of stomachs and small intestines, in blood plasma and in samples of colostrum and milk representing different stages of lactation.

The binding of vitamin B_{12} and folate in milk may be of direct nutritional advantage to the young animal, but the influence of the large excess of unsaturated binder-proteins on the intestinal micro-organisms may be more important. Uptake of cyanocobalamin by pure cultures of intestinal bacteria was inhibited by the presence of sow's milk (Ford, 1974). In order to test whether unsaturated binder-proteins exert a similar influence in the intestine of the suckling piglet, [G-^{3}H]cyanocobalamin was added to samples of contents taken from different regions of the small intestine. These were then centrifuged and the distribution of the added vitamin between the two phases, 'solids' and 'supernatants', were determined.

The numbers of bacteria present in some of the samples were determined by microscopy, by direct counting. In addition, sixty-eight bacterial species were isolated, representing the most abundant species in the small intestine of a 3-week-old suckling piglet, and these were tested for their capacity to take up cyanocobalamin and folic acid.

MATERIALS AND METHODS

Experiments on the uptake of [^{58}*Co*]*cyanocobalamin*

Expt 1. A Large White sow of the herd maintained at the Agricultural Research Council Institute for Research on Animal Diseases was taken immediately after farrowing, and transferred with her litter of ten piglets from the piggery to a pen in an isolation compound. Two of the newborn piglets were each given by stomach tube

10 ml sow's colostrum to which had been added 0·5 μg [^{58}Co]cyanocobalamin containing 0·5–1·0 μCi ^{58}Co. Another two piglets were dosed with [^{58}Co]cyanocobalamin diluted with non-radioactive cyanocobalamin to a total of 10 μg. The piglets were returned to the sow for 6 h and were then briefly removed and their whole-body radioactivity determined (see p. 474). The determinations were repeated at intervals for a period of 8 or 16 d. The piglets were then stunned electrically and killed by exsanguination; a sample of the blood (100 ml) was transferred to a heparinized beaker. The bodies were dissected and the distribution of radioactivity among gut, carcass, liver, spleen, kidneys and brain was determined. The livers were weighed and minced, and a sample of each was taken for microbiological assay of vitamin B_{12}.

The remaining six piglets of the litter were similarly dosed at 7 d of age, three with 0·5 μg and three with 10 μg radioactive cyanocobalamin, and subsequently treated similarly to their four litter-mates that had been dosed shortly after birth.

Expt 2. Another newly farrowed sow was transferred to a pen in the isolation compound, with her litter of twelve piglets. One piglet was then given by stomach tube 10 ml sow's colostrum to which had been added 10 μg [^{58}Co]cyanocobalamin containing 0·5–1·0 μCi ^{58}Co, and returned to the sow for 6 h before being removed for determination of whole-body radioactivity. The determination was repeated 1, 5 and 7 d after dosing, when the piglet was killed, and analysed for distribution of radioactivity within the body as described previously.

Eight piglets were similarly dosed, two each at 7, 14, 21 and 28 d of age, 'counted' (for whole-body radioactivity) over the following 7 d and then killed and analysed for the tissue distribution of radioactivity. The three piglets remaining were dosed at 49 d of age and 'counted' over 7 d. One was then killed and analysed for the tissue distribution of radioactivity, and two were given a second dose of 10 μg radioactive cyanocobalamin and 'counted' over a further 7 d before being killed and analysed for the tissue distribution of radioactivity.

The following procedures were also carried out. The stomach and the entire small intestine were quickly removed. The stomach was opened and its contents centrifuged at 30000 *g* for 30 min at about 2°, and the supernatant fluid was decanted through Kleenex tissue. The stomach mucosa was scraped off as completely as possible, after being washed free from particles of milk curd. The scrapings were gently homogenized with Tyrode's solution (Tyrode, 1910) and frozen to −30°.

The small intestine was freed from the mesentery and divided into three approximately equal lengths. The contents of each length were washed out with 250 ml ice-cold Tyrode's solution and gently homogenized. A 100 ml sample of this suspension was used immediately in tests on uptake of added cyanocobalamin and folic acid into intestinal bacteria. A further 100 ml sample was centrifuged at 30000 *g* for 10 min. The residue was resuspended and diluted to 100 ml with Tyrode's solution, and used in parallel tests to determine uptake of added vitamins in the absence of the supernatant intestinal fluid.

A portion of this supernatant fluid was stored at −30° and later tested for vitamin B_{12}-binding capacity and IF activity.

The three lengths of emptied intestine were opened and the mucosal surface carefully washed with Tyrode's solution and drained. The mucosa were scraped off and weighed, and then homogenized after the addition of Tyrode's solution and frozen to $-30°$.

From one of the piglets aged 21 d at slaughter, samples of the contents of the upper, middle and lower small intestine were taken and serially diluted in MRS broth (de Man, Rogosa & Sharpe, 1960). These dilutions were inoculated onto MRS agar plates, which were incubated anaerobically for 48 h at 37°. Representative colonies were taken from plates at the 10^4–10^8 dilutions, and transferred to MRS broth. From their appearance on microscopic examination and their Gram staining characteristics, and from growth tests in selective media, the isolates were provisionally identified as being predominantly lactobacilli, streptococci and coliforms, with some staphylococci. Sixty-eight of the isolates were established in pure culture in chemically defined media (Ford, 1974) for tests on cyanocobalamin and folic acid uptake.

In a further experiment, piglets were taken from a litter at parturition, and at 1, 2, 4, 7 and 13 d of age. They were killed and their livers, stomachs and samples of blood plasma were taken for examination. The livers and blood plasma were assayed for vitamin B_{12} and folate contents, and the blood plasma was assayed also for unsaturated capacity to bind the two vitamins. Extracts of the stomach mucosa were prepared as described previously, and their capacity to bind added cyanocobalamin was determined. The experiment was discontinued at 13 d when the sow became infected with mastitis. The results are of interest for comparison with those obtained for older piglets in Expt 2, and they are included in Table 5 as relating to 'litter 1'.

Further treatment of the homogenized mucosas of stomachs and small intestines

The frozen preparations were thawed, and the mucosal debris further disrupted by treating the brei in a cooled cell with an ultrasonic probe (Soniprobe type 7530A; Dawe Instruments Ltd, London), for 1 min at the maximum power setting. The sonicate was centrifuged at 30000 ***g*** for 30 min at 2°, and the supernatant fluid was filtered through Whatman No. 42 paper.

Measurement of vitamin B_{12}*-binding capacity*

The capacity of samples of blood plasma and milks, and extracts of mucosas and intestinal contents, to bind added cyanocobalamin was measured as follows. To 1 ml portions of test sample were added graded amounts of [G-^{3}H]cyanocobalamin in 1 ml water. A 1 ml sample of each mixture was then transferred to a sac of 8 mm dialysis tubing and dialysed for 48 h at 4° against eight successive 100 ml portions of buffer solution, pH 7·0, containing 0·15 M-sodium chloride and 0·02 M-sodium phosphate. The residual [G-^{3}H]cyanocobalamin in the sacs was measured by use of a liquid scintillation analyser (Type PW 4510/01; N. V. Philips Gloeilampenfabrieken, Eindhoven, The Netherlands). Alternatively, and more simply, the binding activity was measured by adding excess [G-^{3}H]cyanocobalamin to a sample of the test preparation and separating free and bound [G-^{3}H]cyanocobalamin by filtering the mixture

through a column of Sephadex gel G-25 (Pharmacia Ltd, Uppsala, Sweden). Results obtained by the two methods were in fair agreement.

Binding of [G-^{3}H]cyanocobalamin added to intestinal contents

To 5 ml portions of the ice-cold diluted intestinal contents (see p. 471) were added 1, 2, 5 and 10 ng [G-^{3}H]cyanocobalamin in 1 ml Tyrode's solution. The mixtures were allowed to stand for 10 min at room temperature, with occasional stirring, and centrifuged at 5000 *g* for 15 min. The supernatant fluids were decanted and the residues taken up in 2 ml cold Tyrode's solution. These suspensions were centrifuged again and the supernatant fluids decanted. The washed residues were dissolved in 1 ml hyamine hydroxide and the solutions transferred to scintillation vials, and counted after addition of 10 ml scintillator solution.

A parallel series of tests was done with the intestinal contents from which the original aqueous phase had been removed by centrifugation and substituted by Tyrode's solution (see p. 471).

Microbiological assay of vitamin B_{12} *and folate*

Vitamin B_{12} activity in colostrum, milk, blood plasma and liver was measured using *Lactobacillus leichmannii* ATCC 4797, after digestion of the test samples with cyanide-activated papain, as described by Gregory (1954). Folate activity was measured using *Lactobacillus casei* ATCC 7469 as described by Ford *et al.* (1972).

Milk samples

Colostrum was obtained from two Large White sows by manual expression during farrowing. Samples of milk were obtained from the same animals at intervals during lactation, as described by Braude, Coates, Henry, Kon, Rowland, Thompson & Walker (1947).

Measurement of IF activity

Tests for IF activity in sow's milk and in extracts of mucosas and gut contents were done by the method of Ardeman & Chanarin (1968), modified as described by Wangel & Callender (1965). The method is based on the fact that antibody to IF is present in the blood of about 40% of patients with pernicious anemia and destroys the ability of IF to bind vitamin B_{12}; IF is measured as the change in binding activity that results from pre-addition of serum containing specific antibody to the test sample. Schwartz (1967) suggested that antibody against human IF may be used to assay porcine IF.

Labelled vitamins

[^{58}Co]cyanocobalamin, [G-^{3}H]cyanocobalamin and [G-^{3}H]folic acid (potassium salt) were purchased from the Radiochemical Centre, Amersham, Bucks. They were diluted with non-radioactive cyanocobalamin and folic acid to convenient concentrations of the labelled compounds.

Determination of whole-body radioactivity

Piglets up to 3 weeks old were placed in an open-topped wooden box of length 0·375 m, height 0·45 m and of variable width: 0·125, 0·15, 0·175 or 0·20 m; different widths were used to restrain piglets of different sizes. The box was placed symmetrically between two horizontally opposed scintillation detectors of the Compton whole-body counter (Sansom, Taylor, Wheelock & Vagg, 1971), with their faces 0·2 m from the vertical sides of the box, and their length 0·5 m, overlapping the box equally at both ends. The system was standardized and corrections were made for the radioactive decay of ^{58}Co by counting, in the same way, a standard consisting of 0·5–1·0 μCi [^{58}Co]cyanocobalamin ('one piglet' dose) in 1 l saline containing 1 mg stable cyanocobalamin. The rate for 1 μCi counted in this way was about 2500 counts/s (efficiency 6·7 %) and the background count rate was about 275 counts/s. These high count rates made it possible to obtain good statistical accuracy (SD > 1 %) with a counting period of 100 s, during which the piglets remained still and quiet.

Piglets more than 3 weeks of age were 'counted' in a similar way, using the method of restraint described by Beer, Sansom & Taylor (1974).

Each measurement of the whole-body radioactivity for an individual piglet has been expressed as a percentage of that piglet's whole-body radioactivity 6 h after dosing with [^{58}Co]cyanocobalamin.

RESULTS

Expt 1. Retention of ^{58}Co given orally as [^{58}Co]cyanocobalamin to piglets at birth and at 7 d of age

Table 1 gives the dosing schedule, and the percentage of the test doses of ^{58}Co retained by the piglets at different times after dosing. It is assumed that retention of radioactivity may be equated with the retention of cyanocobalamin. It was planned to give test doses of 0·5 or 10 μg cyanocobalamin in 10 ml sow's colostrum, but in several instances some of the dose was lost by spillage during dosing, or by regurgitation afterwards. The extent of these losses was estimated by comparing, 6 h after dosing, the whole-body radioactivity of piglets which had lost some of the dose with mean whole-body radioactivity of the five piglets in which no loss was observed. The radioactivity in these latter piglets was uniform to within $\pm$ 5 % of the mean value.

It is apparent that cyanocobalamin was efficiently retained by the newborn piglets, even when given in a single dose of about 10 μg, equivalent to the amount of vitamin B_{12} in 5 l sow's milk. At this high dosage level there was very little or no loss of isotope by day 7. With the 0·5 μg test dose there was an initial loss of about 12 % of the isotope during the 1st day, and thereafter no further loss by day 7.

The 7-d-old piglets showed more individual variation in their retention of the test dose. For the three animals given the higher dose there was a progressive reduction in the retention of isotope, from about 94 % at day 1 to 65·3–87·6 % by day 7. Retention of the smaller test dose was marginally more efficient. With two of the piglets it averaged 96·8 % at day 1 and 92·5 % at day 7; the third piglet showed a greater decrease, from 91·1 % at day 1 to 73·3 % at day 7.

Table 1. *Expt* 1. *Percentage of test doses of* [^{58}*Co*]*cyanocobalamin, given orally in* 10 *ml colostrum at birth and at* 7 *d, retained by piglets at different times after dosing**

(Values given in parentheses were obtained by interpolation)

Piglet no. ...	121	122	123	124	125	126	127	128	129	130
Age at dosing (d) ...	<0·5	<0·5	<0·5	<0·5	7	7	7	7	7	7
Dose of [^{58}Co]cyanocobalamin (μg)	0·50	0·50	10·0	*c.* 6·7†	*c.* 0·36†	0·50	*c.* 0·36†	*c.* 7·1†	10·0	*c.* 7·4†
Retention (% dose)										
at days: 1	87·8	88·8	98·0	93·7	95·6	91·1	97·9	94·2	96·4	92·4
2	86·9	86·1	98·6	94·9	93·4	94·8	98·4	93·5	95·4	93·6
3	86·1	85·7	97·6	90·2	95·8	96·2	101·7	90·6	—	90·8
4	85·8	88·2	98·1	94·1	93·7	79·9	94·2	71·3	89·4	86·6
6	86·8	92·7	102·7	96·5	93·4	74·2	94·0	66·9	88·6	86·2
7	88·0	92·9	101·4	96·1	(92·1)	(73·3)	(92·8)	(65·3)	(87·6)	(85·1)
8	79·3	83·8	93·2	86·5	—	—	—	—	—	—
9	—	—	—	—	—	—	90·5	62·1	—	—
11	—	81·9	—	82·4	—	—	—	—	—	—
14	—	81·6	—	84·8	83·3	67·1	—	—	80·4	77·1
16	—	77·2	—	81·1	81·7	62·7	—	—	76·1	75·1

* For details of the experimental procedures, see pp. 470–1.

† Some of the test dose of 0·5 or 10 μg [^{58}Co]cyanocobalamin was lost either by spillage during dosing, or by regurgitation after dosing; see p. 474.

Table 2. *Expt* 1. *Distribution of radioactivity* (% *retained dose*) *at slaughter within the body of piglets which had been given test doses of* [^{58}*Co*]*cyanocobalamin orally in* 10 *ml colostrum at birth and at* 7 *d**

Piglet no.	Carcass	Liver	Kidneys	Muscle (/kg)	Spleen	Brain	Total liver vitamin B_{12} (μg)†	[^{58}Co]cyanocobalamin retained in liver (μg)	Ratio, retained dose: total liver vitamin B_{12}
121	93·9	56·7	1·64	—	0·38	—	—	—	—
122	94·4	67·6	2·71	15·7	0·40	1·28	11·7	0·26	0·022
123	94·5	56·1	1·61	—	0·50	—	—	—	—
124	93·2	55·5	3·09	11·6	0·16	1·96	12·6	3·02	0·240
125	98·6	54·0	2·71	11·3	0·34	1·38	19·9	0·15	0·008
126	94·3	52·0	4·77	23·9	0·40	2·89	12·8	0·16	0·013
127	92·6	50·8	2·52	11·9	0·40	1·18	13·8	0·16	0·012
128	90·3	58·5	4·67	27·7	0·43	2·85	10·1	2·57	0·255
129	95·3	53·4	3·93	16·8	0·35	1·51	23·1	4·06	0·176
130	93·1	53·5	3·24	19·6	0·35	1·64	21·9	2·97	0·136

* For details of the experimental procedures, see Table 1 and pp. 470–1.
† Determined by microbiological assay; for details, see p. 473.

Comparing the values for average retention of cyanocobalamin given at birth or at 7 d of age, it is evident that closure of the gut was not associated with any considerable decrease in retention.

Distribution of the retained [^{58}Co]cyanocobalamin within the body

In a study of the uptake of cyanocobalamin in 8–10-d-old suckling rats, Gallagher & Foley (1971) found that the intestinal epithelium was capable of taking up much larger amounts of the vitamin than were transported into the circulation. In the present experiments it appeared that a dose of 10 μg cyanocobalamin was well retained by the suckling piglet, but it remained to be shown that this retained vitamin was not still largely within the gut mucosa, since the rate of renewal of the intestinal epithelium during the suckling period may be considerably slower than after weaning (Koldovsky, Sunshine & Kretchmer, 1966).

Table 2 gives the distribution of the retained isotope within the bodies of the piglets. The animals were killed immediately after the final determination of whole-body radioactivity, 8 or 16 d after dosing, and the entire gut was excised. The amount of radioactivity remaining in the carcass was determined and calculated as a percentage of the whole-body radioactivity. The difference, which varied from 1·4 to 9·7% (average 6·0%), was the content of isotope in the gut and the blood, of which the amount in the blood was undetectably small. Most of the radioactivity was in the livers, which contained on average 55·8% (range 50·8–67·6%) of the whole-body radioactivity. Much of the remaining radioactivity was in muscle tissue which contained, per kg, 11·3–27·7% (average 17·3%). The content in kidneys, brain and spleen averaged 3·10, 1·84 and 0·37%, respectively.

In the animals given approximately 10 μg [^{58}Co]cyanocobalamin, the amount retained contributed a substantial proportion of the total vitamin B_{12} in the liver.

Expt 2. Retention of [^{58}Co]cyanocobalamin given orally to piglets at birth and at intervals to 56 d of age

Table 3 gives the dosing schedule, and the percentage retention of the test dose when given at graded time intervals up to 56 d. In the previous experiment the piglets dosed at birth retained about 90% of the 10 μg test dose after 7 d; in this experiment only one piglet was dosed at birth and its retention at 7 d was lower, at 66·6%. Two piglets dosed at 7 d of age retained 88·8 and 62·2% (average 75·5%). The two dosed at 14 d gave widely different values; one retained 56·3% and the other retained only 2·6%. At 21, 28, 49 and 56 d the average retention values (%) were broadly similar at 23·0, 20·3, 20·5 and 18·6 respectively, with extremes at 10·0 and 31·5. In all these piglets, 21-d-old or more, the whole-body radioactivity decreased rapidly after dosing and at 3 d or earlier was little greater than at 7 d. Retention in the newborn and 7-d-old piglets, in this experiment as in the preceding experiment, was markedly greater. The general picture is of a sharp reduction in the efficiency of retention of the 10 μg test dose, occurring between 7 and 21 d of age.

The extremely poor retention (2·6%) of [^{58}Co]cyanocobalamin by piglet no. 322

Table 3. *Expt 2. Percentage of a test dose of [^{58}Co]cyanocobalamin, given orally in 10 ml colostrum at birth and at intervals to 56 d, retained by piglets at different times after dosing**

Piglet no. ...	314	315	316	322	326	321	325	323	318	327	317		319	
Age at dosing (d) ...	<0·5	7	7	14	14	21	21	28	28	49	49	56†	49	56†
Dose of [^{58}Co]-cyanocobalamin (μg)	*c.* 8‡	10	10	10	10	*c.* 7‡	*c.* 7‡	10	10	10	10	10	*c.* 5‡	10
Retention (% dose)														
at days: 1	81·5	105·0	96·2	83·6	103·3	42·8	98·5	96·4	96·6	45·9	46·7	25·6	70·2	24·3
2	—	103·1	92·2	10·7	97·9	27·4	58·0	11·8	37·3	30·3	13·9	21·1	32·9	21·4
3	—	—	—	—	—	21·0	29·7	11·2	34·5	—	—	—	—	—
5	80·9	101·0	64·4	4·8	71·1	19·3	27·4	9·9	30·3	29·8	13·2	17·6	31·7	20·0
6	—	99·9	61·6	3·4	62·3	—	—	—	—	—	—	—	—	—
7	66·6	88·8	62·2	2·6	56·3	19·7	26·3	10·0	30·6	28·2	12·8	17·8	31·5	19·3

* For details of the experimental procedures, see pp. 470–1.

† Piglets 317 and 319 were dosed at 49 d and again at 56 d. In calculating retention of the second dose, a correction was applied for the residual radioactivity from the first dose.

‡ Some of the test dose of 10 μg [^{58}Co]cyanocobalamin was lost either by spillage during dosing, or by regurgitation after dosing; see p. 474.

Table 4. *Concentrations of vitamin B_{12} and folate (ng/ml), and unsaturated binding capacity (ng/ml) of sows' colostrum and milk**

	Vitamin B_{12}		Folate	
	Concentration	Unsaturated binding capacity	Concentration	Unsaturated binding capacity
Sow no. 27				
Colostrum	1·9	43	13·4	167
Milk: day 2	1·0	530	3·8	218
8	1·9	143	4·1	96
16	1·6	61	2·6	86
Sow no. 149				
Colostrum	5·5	44	22·2	140
Milk: day 4	9·9	643	12·8	82
9	5·1	177	6·6	63
18	4·7	133	7·3	53

Mean values for 12 sows	Mean	SD
Colostrum	1·52	1·12
Milk: day 7	1·70	1·12
14	2·41	1·89
21	1·46	0·77
28	1·40	0·76
35	1·46	0·79
42	1·51	0·85
49	1·64	0·91

* For details of the experimental procedure, see p. 472.

cannot be explained in terms of any other abnormality. Most of the test dose left its body during the 2nd day after dosing, but the animal was apparently in good health and not scouring. *Post mortem* the gut appeared normal.

Distribution of the retained [^{58}Co]cyanocobalamin within the body

The piglets were killed 7 d after dosing in this experiment. The pattern of distribution of the retained radioactivity was similar to that in Expt 1, but the content in the livers averaged only 36·0% (range 29·9–49·6%) compared with 55·8% in Expt 1. The content of isotope in the gut and blood together averaged 13·1% compared with 6·0% in Expt 1.

Content of vitamin B_{12} and folate, and unsaturated binding capacity of sow's colostrum and milk

Table 4 gives the content of vitamin B_{12} and folate, and of the unsaturated binding capacity for these two vitamins in samples of colostrum and milk from two Large White sows. It also gives mean values for the vitamin B_{12} content in samples of colostrum and milk taken from twelve sows at parturition and at weekly intervals to 7 weeks.

In the present experiments, the average intake of milk/piglet during the 1st week of life would have been about 500 ml/d, increasing to a maximum of about 700 ml/d by the 4th week (cf. Barber, Braude & Mitchell, 1955). Thus, intake of vitamin B_{12} with

Table 5. *Expt 2. Concentrations of vitamin B_{12} and folate in livers and blood plasma of suckling piglets, and unsaturated binding capacity (UBC) of the plasma**

(Values given in parentheses represent cyanocobalamin retained from the test of [^{58}Co]cyanocobalamin (see p. 471))

Piglet no.	Age (d)		Vitamin B_{12}				Folate			
			Liver		Plasma		Liver		Plasma	
	Litter 1†	Litter 2	μg/g	μg/liver	ng/ml	UBC (ng/ml)	μg/g	μg/liver	ng/ml	UBC (ng/ml)
1	<0·5		0·30	4·2	2·1	1·7	0·44	6·3	17	6
2	1		0·43	5·0	0·4	—	0·93	10·8	28	—
3	2		0·34	7·0	0·4	2·2	1·21	25·0	36	13
4	4		0·20	8·2	0·4	1·2	1·03	41·5	23	26
5	7		0·17	6·2	0·3	1·2	1·37	50·1	25	26
6	13		0·08	6·4	0·2	1·5	0·60	51·0	12	38
314		7	0·11	6·7 (1·8)	0·10	1·0	0·59	37·0	11	35
315		14	0·11	9·2 (2·6)	0·11	0·9	0·70	56·3	12	13
322		21	0·09	10·8 (0·13)	0·16	0·9	0·57	70·4	13	11
321		28	0·11	18·7 (0·57)	0·12	1·0	0·62	109·6	13	3
323		35	0·13	24·7 (0·30)	0·11	1·5	1·49	242	19	5
327		56	0·12	37·2 (0·89)	0·08	1·2	8·25	2555	19	4
317		63	0·13	53·7	0·08	1·1	8·10	3542	16	3

* For details of the experimental procedures, see p. 473.
† For details, see p. 472.

the milk would have been about 0·8 μg/d during the 1st week and 1·1 μg/d during the 4th week. These values contrast with approximate estimates of the intake of unsaturated vitamin B_{12}-binding capacity during the 1st week, >200 μg vitamin B_{12} equivalent/d, and during the 4th week, about 70 μg vitamin B_{12} equivalent/d.

The folate content of the milks was apparently much lower than that in human milk and cow's milk, which contain about 50 ng/ml (Ford, Salter & Scott, 1969). However, if these low values are taken as typical, it is barely possible to attribute the rapid increase in liver folate (Table 5) to uptake from the milk. The assays were repeated, after predigesting the milk samples with papain, to ensure that no residual unsaturated vitamin-binders could reduce the growth response of the test micro-organisms (Gregory, 1954). The results were unchanged, but they should be accepted with reserve. No such reservation need be made concerning the results for the unsaturated folate-binding capacity. As with vitamin B_{12}, there was a considerable excess of unsaturated binder-protein. From these and other determinations, a typical value for the folate-binding capacity of mature milk was about 80 ng folic acid/ml.

Content of vitamin B_{12} and folate in livers and blood plasma, and unsaturated binding-capacity of the plasma of suckling piglets

Table 5 gives results for thirteen piglets, ranging in age from <0·5 to 63 d. The concentration of vitamin B_{12} in the liver decreased during the 1st week, and thereafter remained fairly constant at about 0·12 μg/g. Total liver vitamin B_{12} increased progressively, from 4·2 μg at birth to 53·7 μg at 63 d. The piglets had access at all times to a creep feed that contained 0·032 μg vitamin B_{12}/g, and from about 4 weeks of age their intake of the vitamin from this source must have been considerably greater than from milk. This increased intake was not reflected in a faster rate of increase in liver content.

The concentration of vitamin B_{12} in the blood plasma, as in the liver, decreased during the 1st week and then remained constant at about 0·1 ng/ml. The results for unsaturated binding capacity showed little difference between piglet no. 1, which was killed at birth before suckling, and older piglets which were taking in about 200 μg vitamin B_{12} equivalent of unsaturated binder-protein/d with the milk. This does not preclude the possibility that unsaturated binder-protein was taken up from the milk into the blood, as the binder-protein might have been removed by the liver and so have failed to appear in systemic blood. Herbert (1958) showed that gastric IF promotes the uptake of vitamin B_{12} in liver slices, and suggested that the liver cells contain receptors that trap circulating IF. It is possible that they might also retain any unsaturated binder-protein taken up from milk.

The folate content in the liver increased from 6·3 μg at birth to 242 μg at 5 weeks. Thereafter the rate of increase accelerated sharply and by 8 weeks the folate content was 2555 μg. This faster rate of increase can be attributed to the creep feed, which contained 0·75 μg folate/g. Unsaturated binding capacity of the blood plasma also increased, from 6 ng/ml in the newborn piglet to about 30 ng/ml at 1 week, and then decreased to 3 ng/ml at 4 weeks.

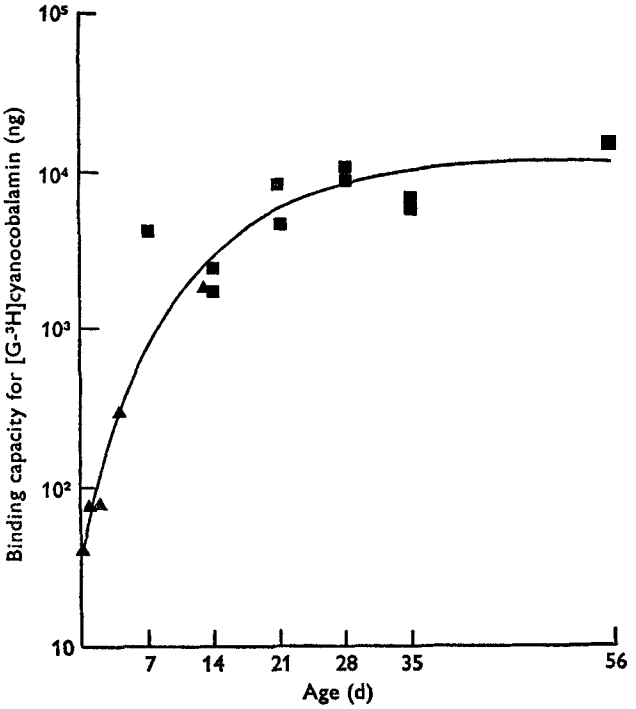

Fig. 1. Increase with age in the capacity of stomach mucosas of individual piglets to bind added [G-^{3}H]cyanocobalamin. For details of the experimental procedures, see pp. 472–3. The animals were from two litters: ▲, litter 1; ■, litter 2.

Development of binding activity in the gut mucosa

Fig. 1 shows that the vitamin B_{12}-binding activity in the stomach mucosa increased with age, from 40 ng at birth to about 2000 ng at 14 d and about 7000 ng at 35 d. Since the capacity to bind vitamin B_{12} is a fundamental property of IF, it is apparent that very little IF was present in the mucosa for several days after parturition.

The standard preparation of porcine IF bound 120 ng [G-^{3}H]cyanocobalamin/mg, and of this activity about 32·5% was attributable to IF. In extracts of the gastric mucosa of piglets aged 7, 14, 21 and 35 d the IF contributed a smaller proportion (12·4, 3·8, 13·6 and 7·9% respectively) of the total binding capacity.

Table 6 gives the unsaturated binding capacity of extracts prepared from the whole stomach mucosas, and from the mucosas of upper, middle and lower small intestine from five piglets aged 7–56 d. For all the animals, unsaturated binding capacity was associated predominantly with the stomach and the proximal one-third of the small intestine. It is not admissible to relate these values to those in Table 3, which gives the percentage retention of 10 μg cyanocobalamin given to the same animals 7 d before slaughter; but it appears that, in the older piglets, the unsaturated capacity of the intestinal mucosa to bind cyanocobalamin was much greater than the capacity of the animal to retain the vitamin.

Porcine gastric IF and vitamin B_{12}-binder in sow's milk were clearly differentiated by their behaviour on gel filtration. By use of a column of Sephadex gel G-200 cali-

Table 6. *Expt 2. Binding capacity (ng [G-^{3}H]cyanocobalamin) of extracts of gut mucosa and gut contents of piglets aged 7–56 d**

				Small intestine					
		Stomach		Upper one-third		Middle one-third		Lower one-third	
Piglet no.	Age (d)	Mucosa	Contents	Mucosa	Contents	Mucosa	Contents	Mucosa	Contents
314	7	4120	291	2596	1001	536	805	208	186
316	14	2482	21	2370	920	270	1364	482	318
322	21	7968	nd	4644	nd	1408	nd	212	nd
323	35	5858	55	7690	289	2648	1238	486	734
327	56	15104	nd	21444	nd	5158	nd	268	nd

nd, Not determined.

* For details of the experimental procedures, see pp. 472–3.

brated with reference proteins of known molecular weight, it was possible to estimate by interpolation a molecular weight of 130000 for IF and 108000 for the sow's-milk binder-protein (cf. Andrews, 1964). The value of 108000 contrasts with an estimate of 55000 given by Gregory & Holdsworth (1955*b*). Burger & Allen (1974) found a similarly wide discrepancy between their estimates of the molecular weight of vitamin B_{12}-binders in human milk and saliva obtained by gel filtration (136000 and 148000) and by sedimentation equilibrium ultracentrifugation and by amino acid and carbohydrate analysis (61000–66000). They explain it as being due to the large content of carbohydrate in the binder-proteins, since glycoproteins are known to give unduly high values for molecular weight when examined by gel filtration. The vitamin B_{12}-binder in sow's milk is also a glycoprotein (Gregory & Holdsworth, 1955*a*, *b*) and the gel filtration value of 108000 is probably too high. However, for the purposes of the present study, gel filtration offered a means for distinguishing the milk binder-protein from gastric IF and other endogenous binder-proteins.

The elution profiles for [G-^{3}H]cyanocobalamin bound to IF or to sow's milk were compared with those obtained for [G-^{3}H]cyanocobalamin added to extracts of intestinal mucosa of piglets of all the ages represented in the study. Although resolution of mixtures of IF and milk binder-protein was poor, it was clear that all mucosal extracts contained both binders. In a further experiment, using preparations from 13-d-old piglets that had been weaned at 2 d of age onto a diet of sterilized cow's milk which had no vitamin B_{12}-binding activity, the stomach mucosas and the aqueous phase of the intestinal chyme gave two peaks of vitamin B_{12}-binder activity, a large peak corresponding closely with porcine IF, and a small peak of apparent molecular weight 72000. There was no peak corresponding to the position of the sow's-milk binder-protein.

Vitamin-binders in bile

Bile contains unsaturated vitamin B_{12}-binder; Gräsbeck (1960) reported that human bile takes up about 1 ng vitamin B_{12}/ml, and porcine bile appreciably more. In the present study, the contents of the gall bladder of a 7-d-old suckling piglet (*c.* 0·5 ml) had unsaturated capacity to bind 155 ng cyanocobalamin, equivalent to about 310 ng/ml. A bulk sample of gall bladder bile from 10-week-old weaned piglets bound 96 ng/ml, but had no detectable IF activity; on filtration using Sephadex G-200, [G-^{3}H]cyanocobalamin added to these specimens was eluted as a symmetrical peak coincident with porcine IF. There was no indication of the presence of milk binder-protein in the bile from the suckling piglets.

The bile from the 10-week-old piglets was also able to bind 13·5 ng added [G-^{3}H]-folic acid/ml.

Influence of bile on uptake of cyanocobalamin by intestinal bacteria

In studies on the flow of digesta in Large White pigs weighing 50–70 kg, Auffray, Martinet & Rérat (1967) found that the secretion of bile between 08.00 and 15.00 hours totalled 470 ml. This large secretion suggests that the bile becomes an important source of endogenous non-IF vitamin B_{12}-binder in the small intestine during

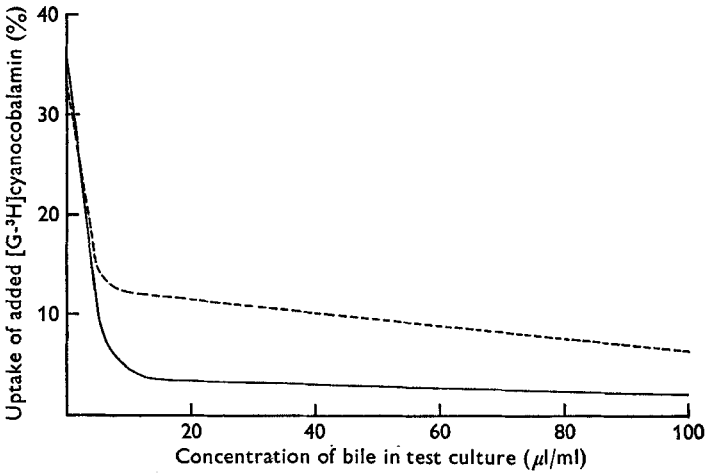

Fig. 2. Influence of bile on uptake of cyanocobalamin by *Escherichia coli* 0101K and *Lactobacillus bifidus* NCDO 1452. Bile from 10-week-old piglets was added to graded concentrations in 5 ml portions of the test cultures: 10 ng [G-^{3}H]cyanocobalamin was then added to each, and the mixture incubated for 1 h at 37°. The cultures were then centrifuged, and uptake of isotope into the cells measured: (– – – – –) *E. coli*; (———) *L. bifidus*.

the early weeks of life. Bile from the 10-week-old piglets was therefore examined for its effect on uptake of [G-^{3}H]cyanocobalamin by *Lactobacillus bifidus* NCDO 1452 and *Escherichia coli* 0101K, both of which take up the vitamin with great avidity (Ford, 1974). Fig. 2 shows the influence of the bile, at graded concentrations in 5 ml portions of the test cultures, on the extent of uptake of 10 ng [G-^{3}H]cyanocobalamin during incubation for 1 h at 37°. With increasing concentration of bile the uptake of cyanocobalamin during the period of incubation decreased, rapidly at first, until the content of bile in the test cultures was sufficient to bind all the added cyanocobalamin, and thereafter more slowly. It is apparent that in presence of bile the extent of uptake of cyanocobalamin into the bacteria was sharply reduced and that *E. coli* took up the bound vitamin more readily than did *L. bifidus*. Much the same was found in tests on the influence of porcine IF on uptake of cyanocobalamin by these same organisms (Ford, 1974).

Removal of the bile by centrifuging the test cultures and resuspending the organisms in fresh medium restored their capacity to take up cyanocobalamin; the influence of the bile was external to the cell, and may be explained in terms of competition for free cyanocobalamin between unsaturated binder-proteins in the bile and in the bacterial cell wall. The relatively larger uptake of bound vitamin by *E. coli* is probably attributable to a higher affinity of its surface receptor sites for the vitamin; Giannella, Broitman & Zamcheck (1972) report widely different vitamin B_{12}-binding constants for different bacteria.

Uptake into 'intestinal bacteria' of cyanocobalamin added to intestinal contents

The chyme in the stomach and throughout the small intestine contained unsaturated binder-protein in the aqueous phase, some of it endogenous and some derived from

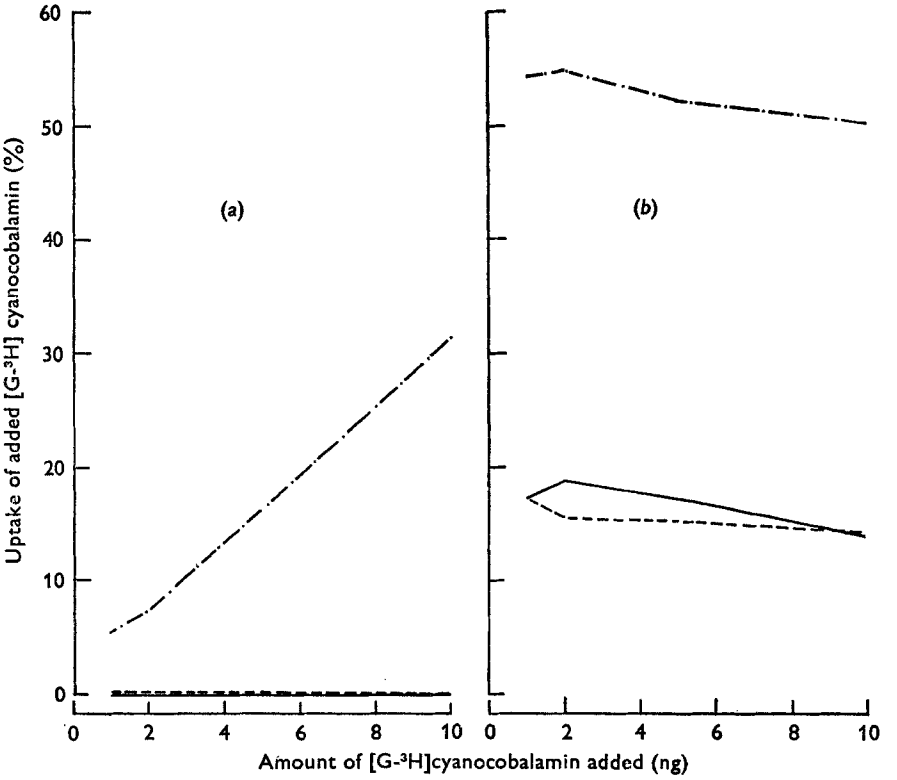

Fig. 3. Influence of the aqueous phase of intestinal chyme on uptake of added [G-^{3}H]cyanocobalamin into the 'solids' phase. The small intestine was taken from a 14-d-old piglet (no. 316) at slaughter and divided into three equal lengths, upper, middle and lower. The contents of each length were washed out with 250 ml Tyrode's solution and gently homogenized. (*a*) To 5 ml portions of homogenate were added 1, 2, 5 and 10 ng [G-^{3}H]cyanocobalamin. The mixtures were then centrifuged and the uptake of radioactivity into the residues was determined. (*b*) A 100 ml portion of each homogenate was centrifuged and the residues resuspended to 100 ml in Tyrode's solution. Portions (5 ml) of this 'washed' chyme were taken, and the uptake of added [G-^{3}H]cyanocobalamin into the 'solids' phase was determined. Segments of small intestine: (———), upper; (– – – –), middle; (–·–·–·–), lower.

milk. The effect of this binder-protein on uptake of added cyanocobalamin by 'intestinal bacteria' was studied.

Fig. 3*a* shows the extent to which added [G^3-H]cyanocobalamin was taken up by and recovered in the 'solids' phase of contents from the small intestine of a 14-d-old piglet (no. 316). Vitamin added to contents of the upper and middle segments of intestine was quantitatively recovered in the aqueous phase. There was no significant uptake into the 'solids' phase, which was largely contributed by bacterial cells with some mucosal debris.

As may be seen from Table 6, the aqueous phase in the contents of the upper segment of small intestine had unsaturated capacity to bind 920 ng cyanocobalamin; the 5 ml portions taken in the present experiment had capacity to bind about 17 ng, more than the 10 ng added at the highest level. Similarly with the contents of the middle segment, the 5 ml portions had capacity to bind about 25 ng. The contents of

Table 7. *Expt 2. Total numbers of bacteria in intestinal chyme from suckling piglets*

Piglet no.	Age (d)	Section of small intestine: Upper one-third	Middle one-third	Lower one-third
314	7	$3{\cdot}7 \times 10^9$	$3{\cdot}3 \times 10^9$	$2{\cdot}9 \times 10^{10}$
315	14	$2{\cdot}1 \times 10^9$	$1{\cdot}8 \times 10^9$	$3{\cdot}7 \times 10^{10}$
316	14	$3{\cdot}5 \times 10^9$	$4{\cdot}6 \times 10^9$	$3{\cdot}9 \times 10^{10}$

the lower segment, however, had capacity to bind only about 5·5 ng in the 5 ml test portions, less than the 10 ng cyanocobalamin added at the highest dose level. This lower unsaturated binding capacity was reflected in the sharply increasing percentage uptake with increase in the test dose (Fig. 3*a*).

In a further experiment the diluted intestinal contents were centrifuged and the supernantant fluid decanted. The residues were resuspended to their original volume in cold Tyrode's solution and tested for their capacity to take up added [G-^{3}H]-cyanocobalamin. The results are shown in Fig. 3*b*. Substitution of Tyrode's solution for the original aqueous phase caused a marked change in the distribution of the added vitamin, much of which was now rapidly taken up by the resuspended residues, more especially those from the lower segment of small intestine, which contained a larger bacterial population. Here the percentage uptake no longer increased with increasing dose; rather it tended to decrease, probably reflecting the operation of saturation kinetics in the binding of cyanocobalamin to the bacterial cells (Giannella, Broitman & Zamcheck, 1971).

These findings for piglet no. 316 were closely similar to those obtained with its 7- and 14-d-old litter-mates, and in all three animals the bacterial populations in the intestinal contents were much the same (Table 7).

From the age of 21 d the intestinal chyme consisted increasingly of undigested food residues and it became impossible to obtain an accurate assessment of the uptake of added [G-^{3}H]cyanocobalamin into the 'solids' phase. The general picture at 21 d was similar to that at 14 d, but with intestinal contents from older animals the efficiency of counting was too low for satisfactory measurements to be made.

Uptake of cyanocobalamin and folic acid by isolated intestinal bacteria

Samples were taken of contents of the upper, middle and lower lengths of the small intestine from one of the 3-week-old piglets (no. 322); they contained $1{\cdot}0 \times 10^7$, $3{\cdot}8 \times 10^8$ and $1{\cdot}2 \times 10^9$ bacteria/ml respectively. The samples were serially diluted and surface-plated onto media appropriate for the isolation of coliforms, streptococci and lactobacilli. Sixty-eight isolates were made and examined microscopically, and of these thirty were taken (ten each of presumed coliforms, lactobacilli and streptococci, representative of different colony types within each species) and tested for their capacity to take up added [G-^{3}H]cyanocobalamin and [G-^{3}H]folic acid, as described by Ford (1974).

All ten coliforms, and one of the ten lactobacilli, took up cyanocobalamin; on

addition of 10 ng [G-^{3}H]cyanocobalamin to 5 ml test culture, 6–7 ng were taken up by the bacterial cells in 1 h at 37°. Under similar test conditions, all the lactobacilli took up 10–25 ng of 50 ng [G-^{3}H]folic acid added, whereas the coliforms took up none. The streptococci took up little or none of either vitamin.

DISCUSSION

The present experiments with piglets showed that a 10 μg test dose of cyanocobalamin was efficiently absorbed from the intestine and retained within the body, whether given before or several days after closure of the gut, which in the piglet occurs within 36 h of parturition. In a paper which first described IF-independent uptake of cyanocobalamin by rats during their first 2 weeks of life, Boass & Hastings Wilson (1963) pointed out close similarities between the absorption of vitamin B_{12} and of intact protein during this period and concluded that they are absorbed by the same mechanism. It is an open question whether during this period vitamin B_{12} must be attached to binder-protein before it is capable of being efficiently taken up from the intestine. But vitamin B_{12} ingested by the suckling rat, or by the piglet or the breast-fed baby, would normally be bound to protein and accompanied by a large excess of unsaturated binder-protein. Recent work by Lecce and co-workers (Lecce, 1973; Lecce & Broughton, 1973) has shown absorption to be a two-phase process, involving first the uptake or 'internalization' of the protein molecule into the epithelial cell, followed by its 'transport' into the blood. 'Closure' was more precisely defined as the time after which cells of the intestinal epithelium no longer internalize macromolecules by pinocytosis. With piglets, this process of internalization continued in the ileal epithelium for as long as 3 weeks after the cessation of transport. So we can imagine that, during this period, protein-bound vitamin B_{12} and folate might be efficiently taken up into the mucosal cells and the vitamins there released for transport into the circulation.

If the vitamin B_{12}-binder in milk does act as a carrier in absorption of the vitamin from the intestine, its function would seem to be particularly appropriate to the first 1–2 weeks of life. Certainly the present study indicates that milk-bound vitamin B_{12} was highly available to the piglet in the early days of life before IF appeared in the gut and the adult mechanism became predominant in the regulation of uptake. Williams & Spray (1968) reported similar findings for suckling rats given 0·25 ng [^{58}Co]-cyanocobalamin/g body-weight, and Gallagher & Foley (1971) showed that the high efficiency of uptake by 8–10-d-old rats did not diminish when the test dose was increased from 1 ng to 10 μg. They found, however, that transport of the absorbed vitamins involved a saturable mechanism, and that most of the vitamin absorbed from the larger test doses was eventually returned to the intestine. In the present experiments a 10 μg test dose was transported with the same high efficiency as a 0·5 μg dose (Table 1); so if a similar saturable mechanism operated in the piglets its capacity presumably exceeded 10 μg.

In the rat, the capacity to absorb vitamin B_{12} decreases sharply with the development of IF-mediated absorption (Boass & Hastings Wilson, 1963) and the present

results indicate that the same is true for the piglet. The development of IF-mediated absorption in the rat coincides with marked changes in the gut, including loss of the ability to absorb intact proteins and onset of secretion or increase in the rate of secretion of several digestive enzymes. All these processes are apparently controlled by hormones of the adrenal cortex, and can be stimulated into precocious development with adrenocorticotrophic hormone or cortisone (Halliday, 1959; Gallagher & Foley, 1972). It is an interesting question whether stress associated with premature weaning might similarly promote early maturation, and if so whether this might be of any disadvantage to the young animal. Halliday (1959) found that removal of suckling rats from their mother for 48 h decreased their ability to absorb antibodies and a similar effect resulted from administration of cortisone.

Intake of large amounts of unsaturated vitamin B_{12}- and folate-binder with the milk continues throughout lactation, and strongly reinforces the developing endogenous binder-protein systems. This prompts speculation on the influence of the vitamin-binders on the ecology of the intestinal microflora.

A stable microflora rapidly develops in the intestine of the newborn infant and it consists predominantly of lactobacilli (Haenel, 1970). In the newborn piglet the alimentary tract becomes flooded with very large numbers of coliforms, clostridia, streptococci and lactobacilli, but by 48 h the population has decreased sharply and consists mainly of lactobacilli, as in the breast-fed baby (Williams Smith & Jones, 1963). Williams Smith & Jones (1963) suggest that this is an effect of the low pH of the stomach contents. It is doubtful whether there is any considerable secretion of acid into the stomach of the piglet for several weeks after birth (cf. Jones, 1972); but assuming that the pH of the stomach contents affects the composition of the intestinal microflora, there are probably other determining factors, such as the nutrient requirements of the competing microbial species, and hence the availability of these nutrients.

Uptake of folic acid by folate-dependent lactobacilli and streptococci, and of cyanocobalamin by several species of intestinal bacteria, was suppressed by addition to the cultures of milk containing the unsaturated vitamin-binders (Ford, 1974). In the present study, unsaturated vitamin B_{12}-binder in the intestinal chyme prevented uptake of added cyanocobalamin by the bacteria (Fig. 3*a*). Clearly, the presence of unsaturated vitamin-binders in the intestine would tend to hinder the proliferation of dependent species of bacteria. Bullen, Rogers & Leigh (1972) found what appears to be an analogous situation, namely that unsaturated iron-binding proteins in milk reduce the growth of a pathogenic strain of *E. coli* in vivo and in vitro, and the bacteriostatic effect is abolished by saturating the binder-proteins with Fe or by providing available Fe in the form of haematin.

Bottle-feeding causes changes in the composition of the infant's intestinal flora, and increased susceptibility to gastroenteritis (cf. Haenel, 1970; Bullen & Willis, 1971; Winberg & Wessner, 1971) and it is possible that a factor contributing to these changes is the removal of constraints on the multiplication of micro-organisms that depend on exogenous vitamin B_{12} or folate for their growth. Thus, heat-treatment of the milk would denature its unsaturated folate-binder and release bound folate in a form readily available to intestinal bacteria; in consequence, the availability of folate to

the infant could be reduced and the growth of folate-requiring bacterial species encouraged.

Folate is probably of greater direct nutritional importance than vitamin B_{12} to the milk-fed infant. Prolonged consumption of goat's milk, which is poor in folate, causes folate-deficiency anaemia (Becroft & Holland, 1966) and blood folate concentrations are higher in breast-fed than in bottle-fed infants of the same age (Matoth, Pinkas & Sroka, 1965), probably because the folate in milk is readily destroyed during heat treatment. Ghitis (1966) recommends that folic acid should be added to processed milks intended for infant feeding, and some of the more sophisticated commercial formulations are now supplemented in this way. The logic of the present argument is that to provide readily available folate in this way might encourage overgrowth of folate-requiring bacteria; whether this could be detrimental is another question.

The concentration of folate in sow's milk was surprisingly low, and similar to that in goat's milk. Unlike the piglet, the newborn kid receives a considerable endowment of folate with the colostrum, and from about 3 weeks of age bacterial synthesis in the rumen makes a further contribution (Ford *et al.* 1972). It may be that, in the piglet, folate synthesized by intestinal bacteria makes a significant contribution to the nutrition of the host animal; certainly the rapid increase in liver folate in relation to the small content in the milk supports this possibility. Diet-induced folate deficiency in germ-free animals was remedied by inoculation with folate-synthesizing bacteria (Miller & Luckey, 1963; Coates, Ford & Harrison, 1968), and mice given a diet lacking in folate carried many more folate-synthesizing bacteria (Klipstein & Lipton, 1970). It is possible therefore that the unsaturated binder-protein in milk, in acting to sequester the milk folate and reduce the growth of folate-dependent bacteria in the gut, would encourage the growth of bacteria that contribute to the folate nutrition of the host.

This speculation fits into the general concept that vitamin B_{12}- and folate-binders may profoundly influence the ecology of the gut microflora and the well-being of the suckling animal. But our present understanding of the role of these binder-proteins is overlong on hypothesis and short on known facts; the hypotheses are easier to formulate than to put to the test. We need comparative studies with suckling and early weaned animals, designed to isolate the effects of the binder-proteins on the composition of the gut flora and on the vitamin economy of the host animal, under varied conditions of rearing. Milk contains an assortment of immunological and other antibacterial mechanisms, and it would be naive to assume that any one is uniquely important or indispensable. Ifekwunigwe & Jelliffe (1974) warn against a kind of 'tunnel-vision simplicism' that sees the prospect of a single solution to manifold problems in the formulation of milk substitutes. So, in urging the need for further study of the vitamin-binders in milk, it seems prudent to dismiss any notion that they might prove a panacea. They may be of only marginal physiological importance, and they would in any instance be difficult to conserve in the manufacture of milk substitutes. But their study may provide important information concerning host resistance factors in milk.

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